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INVITED REVIEW

Traditional and emerging roles for the SLC9 Na^+/H^+ exchangers

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Abstract The SLC9 gene family encodes Na^+/H^+ exchangers (NHEs). These transmembrane proteins transport ions across lipid bilayers in a diverse array of species from prokaryotes to eukaryotes, including plants, fungi, and animals. They utilize the electrochemical gradient of one ion to transport another ion against its electrochemical gradient. Currently, 13 evolutionarily conserved NHE isoforms are known in mammals [22, 46, 128]. The SLC9 gene family (solute carrier classification of transporters: www.bioparadigms.org) is divided into three subgroups [46]. The SLC9A subgroup encompasses plasmalemmal isoforms NHE1-5 (SLC9A1-5) and the predominantly intracellular isoforms NHE6-9 (SLC9A6-9). The SLC9B subgroup consists of two recently cloned isoforms, NHA1 and NHA2 (SLC9B1 and SLC9B2, respectively). The SLC9C subgroup consist of a sperm specific plasmalemmal NHE (SLC9C1) and a putative NHE, SLC9C2, for which there is currently no functional data [46]. NHEs participate in the regulation of cytosolic and organellar pH as well as cell volume. In the intestine and kidney, NHEs are critical for transepithelial movement of Na^+ and HCO_3^- and thus for whole body volume and acid–base homeostasis [46]. Mutations in the *NHE6* or *NHE9* genes cause neurological disease in humans and are currently the only NHEs directly linked to human disease. However, it is becoming increasingly apparent that members of this gene family contribute to the pathophysiology of multiple human diseases.

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Introduction

NHEs are ubiquitous ion transporters present across species from simple prokaryotes to eukaryotes, including plants, fungi, and animals [128]. Mitchell and Moyle were the first to propose a cation/proton antiport system which would allow mitochondria to extrude sodium and potassium against a highly unfavorable electrochemical gradient [106]. While the molecular identity of the mitochondrial NHE proposed by Mitchell and Moyle remains still elusive, 13 other NHE isoforms have been cloned thus far [18] (Fig. 1). Mammalian NHE transport was first reported in 1976 by Murer et al. in brush border vesicles isolated from rat small intestine and kidney [111]. Since then, studies at the cellular and whole organism level yielded detailed insights into function and regulation of NHEs. Analysis of NHE mutant mice, starting with the description of NHE1 deficient mice in 1997 [34] and the recent discovery of humans with mutations in NHE6 and NHE9, has further deepened our understanding of NHE function. This article summarizes the currently known roles of mammalian NHEs in physiology and pathophysiology. This article expands on a recent review [46] in particular our knowledge about the intracellular NHEs. Due to size limitations, we decided not to include information on NHE structure but excellent reviews of this topic have recently been published [81, 90].

SLC9A family

SLC9A1–NHE1

In 1982, Pouysségur et al. described a growth factor-activated, amiloride-sensitive Na^+/H^+ transporter [137]. Employing an elegant functional complementation approach with a functionally NHE-deficient cell line, this group went on to clone the first

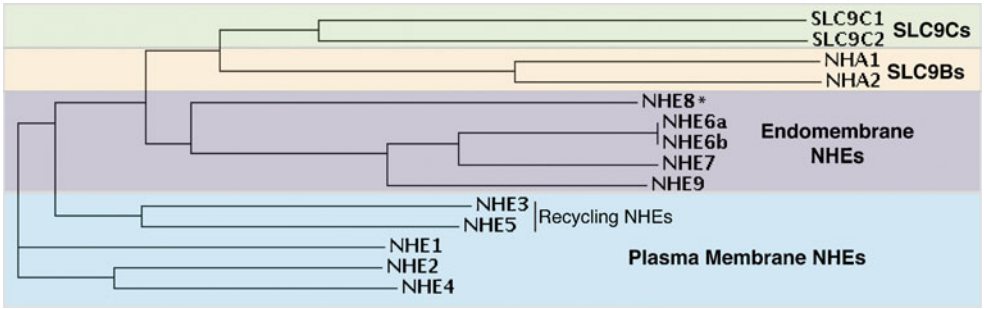

Fig. 1 SLC9 phylogenetic tree. Human SLC protein alignment was completed with Clustal Omega software (Goujon M et al. Nucleic acids research 2010). *NHE8 in specific tissue is also present at the plasma membrane

NHE in 1989 and named it NHE1 [148]. The human NHE1 protein is 815 amino acids long and contains a hydrophobic N-terminal membrane domain of 500 amino acids responsible for NHE transport and a hydrophilic, intracellular 315 amino acid long C-terminus that regulates transport. The mature plasmalemmal NHE1 protein is both N- and O-glycosylated, although glycosylation is not required for transport function [33, 61]. NHE1 is highly sensitive to amiloride, the first NHE1 inhibitor identified [103, 137]. Subsequently, more selective and potent inhibitors have been developed including lipophilic amiloride derivatives (e.g., ethylisopropoylamiloride, EIPA) and benzoylguanidines (e.g., HOE694, cariporide, eniporide) [103]. NHE1 is present in most mammalian cells, notable exclusions are the macula densa, α- and β-intercalated cells of the kidney [16, 133]. Consequently, NHE1 is often referred to as the "housekeeping" NHE isoform. It resides almost exclusively at the surface of cells, but preferentially accumulates in discrete microdomains within the plasma membrane, depending on the type and state of the cell. In polarized epithelial cells, NHE1 localizes to the basolateral membrane, in cardiac myocytes to intercalated disks and T-tubules and in resting fibroblasts to sites of focal adhesions [16, 55, 134]. In contrast, in migrating fibroblasts, NHE1 concentrates at the leading edge of the cell along the border of lamellipodia [40, 41]. NHE1 abundance at the plasma membrane was recently shown to be regulated by direct ubiquitylation of the exchanger [154].

NHE1 activity is a primary cellular alkalinizing mechanism, extruding H^+ derived from metabolism or electrically driven acidification. In addition, NHE1 constitutes a major pathway for Na^+ influx into the cell, which coupled to Cl^- and H_2O uptake, ensures restoration of cell volume following cell shrinkage, a process referred to as regulatory volume increase. In specialized secretory cell types like acinar cells of the parotid or sublingual glands, NHE1 is critical for secretagogue-induced fluid secretion [117, 132]. The unique subcellular localization of NHE1 in certain cell types suggests that NHE1 has additional biological functions to those outlined above. In cardiac myocytes, the specific localization of NHE1 to intercalated disks and T-tubules, but not to peripheral sarcolemmal membranes, is thought to induce pH microdomains affecting the activity of pH-sensitive proteins, such as the gap-junction protein connexin 43 [174] and the ryanodine-sensitive Ca^{++} release channel [182]. NHE1 also participates in cell migration [40, 41, 79, 82, 150]. Inhibition or genetic ablation of NHE1 from fibroblasts significantly reduces migration speed and inhibits chemotaxis. Both ion translocation and anchoring of cytoskeletal proteins through the intracellular NHE1 C-terminus are required [41]. Involvement of NHE1 in cell migration is apparent in vitro in certain cell types (e.g., fibroblasts, MDCK cells); however, there is data to suggest this is not a universal phenomenon. Inhibition of NHE1 transport in granulocytes does not affect chemotaxis and chemokinesis [63]. Furthermore, NHE1 knock-out (KO) mice exhibit normal embryogenesis, suggesting redundant or compensatory mechanisms [15, 34].

Functional regulation of NHE1 is complex and occurs mainly via the intracellular C-terminus. This regulation is conveyed by phosphoinositides, postranslational modifications (phosphorylation, ubiquitylation), and binding proteins. A comprehensive description of these is clearly beyond the scope of this review [13]. The precise structure of NHE1 (or of any other mammalian NHE) is not known, but the structures of two bacterial homologues, NhaA of *Escherichia coli* and NapA of *Thermos thermophilus*, have been determined [71, 88]. A recent review describes the current structural model of NHE1 [90].

The phenotype of two different NHE1 KO mice has been reported. Cox et al. described a spontaneous mutation ("swe"—slow-wave epilepsy) that arose in the Jackson laboratories which resulted in a truncation of the protein in the transmembrane domain with a subsequent NHE-null phenotype [34]. Bell et al. generated a traditional NHE1 KO, achieved by homologous recombination resulting in deletion of NHE1 transmembrane domains 6 and 7 [15]. The loss of NHE1 was compatible with embryogenesis, however, in contrast to the Swe mice the NHE1 KO mice exhibited a decreased rate of postnatal growth and high mortality with only ~10 % of mice surviving 5 weeks after birth [15]. In addition, mice suffered from ataxia and epileptic seizures. This phenotpye was associated with selective neuronal death in the cerebellum and brainstem of KO mice [34]. KO of NHE1 decreased steady-state pH_i, attenuated pH_i recovery from cell acidification (even in the presence of HCO_3^-) and increased expression and current density of voltage-gated Na^+ channels in hippocampal and cortical regions [56, 177, 185]. Thus, loss of NHE1 seems to

alter expression and activity of other membrane transport proteins in the brain, resulting in increased neuronal excitability.

In contrast, increased NHE1 activity is detrimental during episodes of ischemia-reperfusion in cardiac and neural tissues. Enhanced NHE1 activity in these pathological situations causes substantial intracellular Na^+ accumulation. This activates the plasmalemmal Na^+/Ca^{++} exchanger inducing a deleterious increase of intracellular Ca^{++} that triggers various pathways ultimately leading to cell death [74, 77, 98, 99]. Genetic ablation or pharmalogic inhibition of NHE1 during episodes of ischemia-reperfusion mitigated cardiac and neural injuries both in vivo and in vitro in rodents and pigs [32, 62, 85]. Consistent with this, NHE1 overactivity has been linked to cardiac hypertrophy and heart failure [168]. Wakabayashi developed a transgenic mouse that selectively overexpressed a constitutively active NHE1 in the heart [114]. Increased NHE1 activity was sufficient to induce cardiac hypertrophy and ultimately heart failure in the transgenic mice. Clinical trials in humans, however, have not found a benefit to NHE1 inhibition. The GUARDIAN trial did not show an overall benefit for NHE1 inhibition by cariporide in acute coronary syndromes, a small benefit was only observed in the subgroup of patients that underwent high risk coronary artery bypass surgery [21, 164]. Likewise, the NHE1 inhibitor eniporide failed to demonstrate a significant benefit in patients with acute myocardial infarction in the ESCAMI trial [188].

The earliest evidence that NHE1 could play a role in cancer stems from the group of Pouysségur and coworkers. In athymic nude mice, injection of CCL39 hamster lung fibroblasts deficient in NHE1 caused tumors less frequently than CCL39 cells with functional NHE1 [83]. Since these initial observations, numerous studies have examined the role of NHE1 in cancer. NHE1-dependent intracellular alkalinization plays an important role in the development of a transformed phenotype, as inhibition of NHE1 activity prevents this [141]. In breast cancer and leukemic cells, inhibition of NHE1 exerts a protective effect against cancer, inducing apoptosis [142, 143]. A fundamental role for NHE1 in cell migration was mentioned above. In breast cancer cells, serum deprivation activates NHE1 to induce cell motility and invasion [140]. The protons extruded by NHE1 at the leading edge create an acidic environment optimal for the activity of proteinases involved in the degradation of extracellular matrix [25]. Low pH also enhances cell–matrix interactions and cell adhesion at the cell front [157]. Thus, NHE1 promotes tumorigenesis via several mechanisms including: cell proliferation, cell migration, invasion, metastasis, and suppression of apoptosis. There is currently no clinical evidence that inhibition of NHE1 is a useful cancer therapy.

SLC9A2–NHE2

NHE2 was cloned by Donowitz et al. and Orlowski et al. independently in 1993 [167, 172]. The resulting protein, NHE2, is 812 amino acids in length and is O, but not N-linked glycosylated [166]. It has been localized to several organs including the gut, skeletal muscle, kidney, brain, uterus, testis, heart, and lung. In the gut, NHE2 is expressed in stomach, duodenum, ileum, jejunum, proximal, and distal colon [67, 139]. Within the kidney, NHE2 is expressed in the thick ascending limb (as well as the macula densa), distal convoluted tubules, connecting tubules and some thin ascending limbs [28]. When expressed in epithelial cells, it predominantly localizes to the apical membrane [28, 67]. Generation of NHE2 KO mice has provided insight into function of this exchanger. The null mice have reduced viability of gastric parietal epithelial cells and reduced net acid secretion. Interestingly they otherwise lack an overt renal or gastrointestinal phenotype, despite significant NHE2 expression in both these tissues [86, 87, 151]. Given the clear presence of NHE2 activity in the macula densa and distal convoluted tubule [133, 152], more detailed studies on the renal phenotype have been performed. NHE2 KO mice display increased renal and plasma renin levels [59]. Despite this, the null mice do not differ from their WT counterparts with respect to blood pressure, plasma aldosterone levels, renal sodium excretion nor tubuloglomerular feedback responses [86, 95, 151]. Moreover, angiotensin signaling does not appear to alter NHE2 localization or function [60]. Further studies on the role of this isoform in the macula densa are necessary to understand these observations.

NHE2 is clearly abundantly present and functional in the colon [57]. Further, it is the predominant brush border NHE in the colon of birds [45]. This has led to more detailed studies on its role in this tissue. Metabolic acidosis and volume contraction induced by a low NaCl diet both increase intestinal NHE2 expression and activity [72, 96]. Further, NHE2 plays a role in mucosal recovery from ischemia reperfusion. Although, whether NHE2 activity is beneficial or detrimental remains to be determined [107, 108]. A role for NHE2 in salivation responses has also been observed using the NHE2 KO mice [132]. Most recently, NHE2 expression was localized to the pituitary. The absence of NHE2 results in gross histological abnormalities in the pars distalis, suggesting that NHE2 may contribute to the volume regulation and composition of folliculo-stellate cell canalicular fluid [104]. Ultimately, despite significant expression and activity in both bowel and kidney, it appears that the absence of NHE2 can be compensated for. It is likely that NHE3 and NHE8 perform these compensatory roles, at least under some circumstances [6, 51, 69]. Perhaps not surprising, given the relatively benign phenotype for NHE2 KO mice, there is no known human disease ascribed to defects in this transporter [110].

SLC9A3–NHE3

Orlowski et al. and Donowitz et al. cloned NHE3 independently in 1992 [129, 165]. This 825 amino acid protein is

expressed to the greatest extent in the gut and kidney, and to a lesser extent in heart, brain, and lung. In the gut, NHE3 is expressed in stomach, small bowel (all three segments), cecum, proximal, and distal colon [129]. In the kidney, NHE3 localizes to the apical membrane of the proximal tubule and thick ascending limb of Henle's loop [17]. NHE3 is highly resistant to inhibition by amiloride and its derivatives [127]. A prominent mode of NHE3 regulation is via recycling between an endomembrane location and the plasma membrane. A complete review of how this exchanger is regulated is beyond the scope of this review. Suffice to say, there is continued research in this area. Recently significant insights into how the NHERFs and ezrin affect NHE3 activity, the mechanisms underlying angiotensin II mediated activation of the exchanger, the role of SGK signaling and NHE3 lipid interactions on activity have been made [1, 8, 9, 30, 64, 68, 112, 116, 149, 184].

The role of NHE3 in gastrointestinal and renal physiology has been extensively studied, largely through the use of genetically altered mice. NHE3 KO mice display absorptive defects in both intestinal and renal tubular epithelia. NHE3 KO animals have decreased sodium and water reabsorption from all intestinal segments measured [86, 87, 173]. This results in volume depletion and hypotension despite increased renin expression [152]. The contribution of a renal sodium leak in the absence of an intestinal defect to the global KO phenotype has been explored by knocking NHE3 into the intestine and by making a conditional renal knockout [91, 118]. These experiments confirm a contribution of both systems to the significantly decreased circulating volume in the NHE3 KO mice. More recently, NHE3 has been found to play a role in intestinal inflammation. Interferon-γ and TNF-α decrease NHE3 activity causing diarrhea [2, 51, 145]. Consistent with a role in inflammatory bowel disease, NHE3 expression and/or activity is decreased in mouse models and human disease [159, 187].

NHE3 mediates the majority of sodium reabsorption from the proximal tubule. This driving force also induces significant water reabsorption. As one H^+ is extruded in exchange for the influx of Na^+, down its concentration gradient into the cell, NHE3 also participates in the reclaimation of HCO_3^- from the pro-urine. Consistent with this, global and proximal tubular NHE3 KO mice display a metabolic acidosis and alkaline urine [91, 152]. Interestingly, NHE3 has been proposed to mediate NH_4^+ efflux into the tubular lumen, although recent acid loading experiments on the proximal tubular KO mice are inconsistent with this [76, 91]. Given the large amount of sodium and water which is not reabsorbed from the proximal tubule in the absence of NHE3 it is surprising the NHE3 KO mice survive at all. This is best explained by the large decrease in GFR observed in these animals, which is mediated by increased sodium delivery to the macula densa [113, 176].

The compelling evidence implicating NHE3 in osmotic reabsorption of water from the proximal tubule and intestine led to the recent realization that this transporter also mediates transepithelial calcium reabsorption [131, 144]. Consistent with this, NHE3 overexpression in an epithelial cell culture model increased tranepithelial calcium flux in a sodium dependent fashion. Moreover, NHE3 KO mice display an increased fractional excretion of calcium and reduced bone mineral density. Intestinal calcium uptake in NHE3 KO mice was reduced when measured by oral gavage of $^{45}Ca^{2+}$ or as the unidirectional flux of calcium across the duodenum. Detailed studies of net calcium flux across the cecum, demonstrated reduced paracellular calcium flux, in the presence of NHE3 inhibition or on tissue isolated from NHE3 KO mice. Taken together this work clearly implicates NHE3 in calcium homeostasis, likely by providing the driving force for paracellular calcium absorption from renal and intestinal epithelia.

SLC9A4–NHE4

SLC9A4 encodes a 798 amino acid protein, NHE4, which was cloned in 1992 [129]. Overexpression studies in NHE deficient cell lines found NHE activity after hyperosmolar stimulation, or after an acid load in the presence of DIDS [19, 26]. NHE4 is relatively amiloride and EIPA resistant [26] and is expressed in the gut, kidney, brain, uterus and skeletal muscle [129]. In the kidney, NHE4 is expressed in the basolateral membrane of the proximal tubule, but much more abundantly in the basolateral membrane of the thick ascending limb and distal convoluted tubules [27]. In the gut NHE4 is expressed in the stomach, small intestine and colon, where it is present in the basolateral membrane of these epithelia [129, 136]. In the brain, NHE4 expression has been localized to the hippocampus [19].

The generation of NHE4 KO mice has provided insight into the physiological role(s) of this isoform [50]. NHE4 KO mice demonstrate persistent hypochlorhydria in association with histological abnormalities of the stomach, which include reduced parietal and chief cell numbers and increased mucous cells. Colonic NHE4 expression and activity is increased by aldosterone, inferring a role for this exchanger in volume regulation. Although the effect of volume depletion on NHE4 KO mice has yet to be reported [3]. Detailed functional characterization of NHE4 KO mice have implicated the exchanger in acid–base homeostasis. At baseline, NHE4 KO mice display a compensated metabolic acidosis without diarrhea. This is greatly exaggerated by challenging the null mice with an acid load. This perturbation demonstrated a failure for the null mice to increase urinary ammonia and net acid excretion. Further evidence that NHE4 participates in acid–base homeostasis was provided by microperfusion studies on dissected medullary thick ascending limbs, where NHE4 expression and activity was increased by acid loading [20]. Whether

humans with hypochlorhydria or renal tubular acidosis exist due to mutations in NHE4 is unknown.

SLC9A5–NHE5

NHE5 is a 896 amino acid protein encoded by the *SLC9A5* gene. Human NHE5 was cloned in 1995 and maps to 16q22.1 [78]. Expression is greatest in the brain but was also observed in testis, spleen and skeletal muscle [5, 7, 78]. NHE5 shows intermediate sensitivity to amiloride inhibition (less than NHE1 but more than NHE3) [161]. Like its closest homologue NHE3, NHE5 localizes to both the plasma membrane and endomebrane compartments(s) and recycles between them [162]. As a NHE5 KO mouse has not been reported, most of the recent literature focuses on the regulation of activity. NHE5 is regulated by RACK1, PKC, PKA, the actin cytoskeleton, and PI3 kinase [4, 124, 162]. Cell surface expression of NHE5 is altered by the scaffolding protein β-arrestin and secretary carrier membrane protein 2 [42, 160]. The former pathway is dependent upon CK2 mediated phosphorylation of the exchanger [97]. Cultured neurons have provided some insight into the role of NHE5 in the brain. Dentritic spine growth is sensitive to changes in pH induced by plasma membrane expression of NHE5 [43]. Moreover, NHE5 activity maintains endosomal pH, which is required for proper Trk family tyrosine kinase trafficking and possibly neuronal differentiation [44]. Given its prominent localization to the brain and cell culture studies implicating NHE5 activity in neuronal differentiation and function, mutation analysis was performed on DNA from patients with familial paroxysmal kinesigenic dyskinesia. However, no coding mutations were observed [155]. We are unaware of mutations in NHE5 causing human disease.

Intracellular NHEs

In the endocytic pathway, organellar pH gradually decreases from early endosomes to lysosomes, with the latter exhibiting a pH of 4.5–5 [39]. Large pH gradients also exist within the recycling endosomal system of polarized cells [169]. Organellar pH is a critical regulator of enzyme activity, intracellular trafficking, membrane fusion, posttranslational modifications, dissociation reactions of receptor–ligand complexes, and uptake of neurotransmitters [123]. Despite the importance of tightly controlled endomembrane pH, the molecular mechanisms maintaining pH in each organelle remain poorly understood. Organellar pH is a balance between acidification by the V-ATPase, proton-leak pathways and counterion conductances. It is generally accepted that organellar pH is not set by regulation of V-ATPase activity per se but by proton-leak pathways (e.g., NHEs) and counterion conductances (e.g., Cl^- channels) [39, 156]. Due to their distinct localization to specific types of organelles, it is likely that intracellular NHEs play a critical part in the regulation of organellar pH. In support of this, overexpression or loss of intracellular NHEs alters organellar pH [35, 80, 115, 130]. This concept is however challenged by studies where knockdown or loss of individual organelle-specific NHEs had no effect on pH [38, 52, 147]. Although redundance may theoretically explain the latter finding, clear biological effects were nevertheless observed, questioning the sole role of organellar NHEs in endomembrane pH regulation [38, 52]. Since NHEs exchange cations for protons, they not only affect intraluminal H^+ concentration but also the concentration of Na^+ and K^+, depending on ion selectivity. However, very little is known about the regulation of organellar Na^+ and K^+ by cation/proton antiporters at the moment. It is likely that organellar cations participate in the regulation of many processes as does pH [146].

SLC9A6–NHE6

NHE6 resides in recycling endosomes, but has a major plasmalemmal distribution in a few specialized cells. In vestibular hair bundles, for example, both NHE6 and NHE9 are enriched in the plasma membrane where they probably exploit high endolymph K^+ to efflux cytosolic H^+. This mechanism allows the hair cell to remove H^+ generated by Ca^{2+} pumping without ATP hydrolysis in the cell [65]. NHE6 is also highly expressed in the basolateral membrane of osteoblasts, especially in areas of high mineralization, where it helps remove excess H^+ generated by this process [93]. Based on this in vitro study, in osteoblasts, an important role for NHE6 in bone turnover has been postulated.

Our knowledge of how the different intracellular NHEs are sorted to specific organelles is very limited. An important observation in this respect was made regarding the intracellular versus plasmalemmal distribution of NHE6. Yeast-two-hybrid screens revealed that NHE6 binds to the cytoplasmic scaffolding protein RACK1 (receptor for activated C kinase) via its intracellular C-terminus. RACK1, originally identified as an adaptor for activated PKC, is a known scaffold protein that interacts with metabolic enzymes, kinases receptors and ion transporters [123]. The luminal pH of recycling endosomes was elevated in RACK1 knock-down cells, accompanied by a decrease in the amount of NHE6 at the cell surface, without alteration of total NHE6 expression. The underlying mechanisms, including the role of activated PKC, remain unknown. The data however indicate that RACK1 regulates the distribution of NHE6 between endosomes and the plasma membrane and thereby contributes to the maintenance of endosomal pH [121]. In addition to RACK1, NHE6 binds to the angiotensin II receptor subtype AT2 in a ligand-dependent manner [138]. Although angiotensin II is known to regulate NHE activity in several cell types, the NHE isoform has remained elusive [123]. Interestingly,

chronic angiotensin converting enzyme inhibition abolishes NHE overactivity in lymphocytes from patients with essential hypertension and this effect seems to be independent of the ubiquitous NHE1 isoform [47]. Perhaps NHE6 is participating in this capacity?

Another important function of NHE6 was demonstrated in hepatoma HepG2 cells, where NHE6 localizes to recycling endosomes and colocalizes with transcytosing bulk membrane lipids [122]. Knock-down of NHE6 lowered recycling endosomal pH and surprisingly, disrupted the apical canalicular plasma membrane with failure to traffic or maintain apical proteins; these effects were associated with reduced abundance of apical lipids. Thus, in HepG2 cells endosomal NHE6 is important for maintaining the polarized distribution of membrane lipids at the apical surface, the maintenance of apical bile canaliculi and consequently cell polarity [122]. In addition to cell polarity, NHE6 participates in clathrin-dependent endocytosis by alkalinizing clathrin containing early endocytic vesicles [179]. NHE6 co-localizes with clathrin and transferrin and knock-down of NHE6 acidifies endosomes inhibiting transferrin endocytosis, but not substrates endocytosed by non-clathrin dependent mechanisms [179]. This is in contrast to a recent report where knock-down of NHE6 did not alter endosomal pH. Only the simultaneous knock-down of NHE6 and NHE9 resulted in an acidification of endosomes, suggesting redundance of endosomal NHEs in some cells [147].

NHE6 is encoded by the X-chromosome both in mice and humans. Mutations in the *SLC9A6* gene cause three phenotypes in humans: the most common manifestation is X-linked Angelman syndrome, characterized by intellectual disability, microcephaly, epilepsy, ataxia, and behavioral abnormalities [52, 163]; second, an Angelman-like syndrome known as Christianson syndrome [31] and finally a syndrome presenting with corticobasal degeneration and tau deposition with severe intellectual disability and autistic behavior [49]. Nonsense and missense mutations as well as deletions in the *SLC9A6* gene have been found (Table 1) [49, 52, 163]. The functional consequences of the individual mutations are currently unclear. Besides the neurological phenotype of patients with *SLC9A6* gene mutations, little is known about extracranial consequences as they have not been examined extensively. Christianson et al. reported skeletal malformations including a long narrow face, straight nose, square prognathic jaw, large ears, and narrow chest in affected family members [31]. Gilfillan et al. observed a low body mass index in many and hyponatremia and systemic hypertension in one case. Interestingly, this study also found that some female carriers were mentally retarded, had learning problems or dyslexia without evidence for aberrant X-inactivation suggesting either haploinsufficieny or a dominant-negative effect in female carriers.

The neurological phenotype of an NHE6 KO mouse was recently described by Walkley et al. [158]. Mutant mice were born at the expected mendelian ratio and had no obvious phenotype, even at older age. Histologically, loss of NHE6 led to abnormal accumulation of GM2 gangliosides and unesterified cholesterol in late endosomes and lysosomes of neurons in selective brain regions, most notably the basolateral nuclei of the amygdala, the hippocampus, the cerebral cortex and cerebrum. In neurons of these regions, lysosomal β-hexosaminidase activity (the enzyme responsible for GM2 ganglioside degradation) was undetectable. An extensive loss of Purkinje cells was observed in the cerebelli of mutant mice. No cell loss occurred in the cerebrum. In addition, small elevations of hyperphosphorylated tau protein were found in soluble brain fractions of mutant mice. Extensive behavioral testing revealed that these histiological abnormalities result in mild motor hyperactivity and deficits in motor coordination in the mutant mice.

Studying the same KO mouse model, Morrow recently found that NHE6 deficient neurons exhibit overacidified endosomes [153]. This overacidification lead to disrupted endosomal BDNF/TrkB signaling due to hastened TrkB degradation, resulting in defective axonal and dendritic branching of neurons. Thus, loss of NHE6 in mice leads to a neuronal endolysosomal storage disease with cell death as well as more subtle alterations in endosomal signaling pathways that impair the wiring of neuronal circuits. The physiological role of NHE6 in non-neurological tissues remains to be determined.

SLC9A7–NHE7

NHE7, cloned in 2001 by Numata et al., is an intracellular NHE that localizes mainly to the trans-Golgi, although it also traffics to the recycling system and to the plasma membrane [119]. It is ubiquitously expressed with high abundance in brain, skeletal muscle, stomach, pancreas, prostate, pituitary, and salivary glands. NHE7 has multiple C-terminal binding partners, including: SCAMPS, proteins involved in vesicle trafficking, calmodulin, a protein known to bind multiple other plasma membrane NHEs, caveolin, CD44, and GLUT1 [73, 92]. The membrane proximal region of the NHE7 C-terminus is responsible for its trans-Golgi localization. An unusual feature of NHE7 is its insensitivity to amiloride, while it is inhibited by benzamil and quinine. Little is known about the physiological relevance of NHE7 and a NHE7 KO mouse has not been reported. NHE7 overexpression in the MDA-MB-231 breast cancer cell line enhances cell overlay, cell–cell adhesion, invasion, and anchorage-independent growth [125]. Thus, in addition to its still poorly defined physiological function, NHE7 may have a role in tumor biology.

SLC9A8–NHE8

NHE3 and NHE2/NHE3 double KO mice exhibit significant EIPA-inhibitable NHE transport in the proximal tubule. In

Table 1 SLC9 family of Na/H exchangers

Protein *Gene* Name	Tissue distribution and subcellular expression	Human disease associations	KO mouse phenotype(s)
NHE1 *SLC9A1*	Ubiquitous (plasma membrane; basolateral surface of epithelia)	Cancer, ischemia-reperfusion damage, arterial hypertension (?)	Ataxia, growth retardation, seizures, slow-wave epilepsy, increased neuronal excitability, resistant to cardiac ischemia-reperfusion injury and pre-mature death.
NHE2 *SLC9A2*	Stomach, intestinal tract, skeletal muscle, kidney, brain, uterus, testis heart, lung; (plasma membrane; apical surface of epithelia)	?	Reduced viability of gastric parietal cells, hypochlorhydria. Increased renal renin content, impaired recovery of intestinal barrier function.
NHE3 *SLC9A3*	Intestinal tract, stomach, kidney, gall bladder, epididymis, brain; (apical surface and recycling endosomes of epithelia)	Congenital Na^+ diarrhea; Sudden infant death syndrome (?)	Mild-diarrhea, acidosis, impaired acid-base balance and Na-fluid volume homeostasis in kidney and intestine. Renal role confirmed in volume homeostasis confirmed by GI knock-in and renal specific KO. Hypercalciuria, reduced bone mineral density, reduced intestinal calcium absorption. Spontaneous distal colitis due to alteration of gut microbiome.
NHE4 *SLC9A4*	Stomach, kidney, brain; (plasma membrane; baso-lateral membrane of epithelia)	?	Stomach inflammation, hypochlorhydria, gastric necrosis. Defective in NH_4 absorption from renal thick ascending limb.
NHE5 *SLC9A5*	Brain (neurons); (plasma membrane and recycling endosomes/synaptic vesicles)	?	?
NHE6 *SLC9A6*	Ubiquitous (recycling endosomes)	X-linked mental retardation (Angel-man/Christianson syndrome).	Hyper-reactivity, increased susceptibility to pharmacologically induced seizures.
NHE7 *SLC9A7*	Ubiquitous (*trans*-Golgi network and endosomes)	Cancer (?)	?
NHE8 *SLC9A8*	Ubiquitous (mid- to trans-Golgi network) and apical plasma membrane in proximal tubule	?	Reduced mucus secretion, increased susceptibility to mucosal injury, increased bacterial adhesion in colon. NHE3/NHE8 double KO mice have lower blood pressure and lower proximal tubular NHE activity compared to NHE3 KO.
NHE9 *SLC9A9*	Ubiquitous (late recycling endosomes)	Familial autism; attention deficit hyperactivity disorder	?
NHA1 *SLC9B1*	Testis-specific	?	?
NHA2 *SLC9B2*	Ubiquitous (plasma membrane, endosomes)	Essential hypertension, diabetes mellitus (?)	Impaired insulin secretion by β-cells, impaired glucose tolerance
Sperm-NHE *SLC9C1*	Spermatozoa (sperm flagellum)	?	Male infertility, asthenozoospermia
NHE11 (?) *SLC9C2*	?	?	?

A Human, *B* v = variant, *?* means the data is not currently available, *(?)* means the data is not certain

search of an additional NHE in the kidney, Aronson et al. cloned NHE8 by searching EST databases [54]. NHE8 is expressed ubiquitously in mouse tissues at the RNA level with a higher level of expression in the kidney, liver, skeletal muscle, and testis. In plasmalemmal NHE-deficient PS120 fibroblasts, overexpression of NHE8 results in sufficient protein at the plasma membrane for functional characterization [180]. NRK cells endogenously express NHE8 at the plasma membrane. Using the latter cells, Moe et al. demonstrated that NHE8 is sensitive to amiloride [189]. When expressed in

HeLa cells, NHE8 localizes primarily to mid- to trans-Golgi [115]. Silencing NHE8 in HeLa-M cells results in perinuclear clustering of endosomes and lysosomes and disrupted endosomal protein trafficking. Consequently, Bowers et al. proposed that NHE8 is either a negative regulator of inward vesiculation or NHE8 might promote back fusion [84].

In addition to its intracellular location, NHE8 localizes to the apical membrane of the renal proximal tubule and intestine. In kidney, NHE8 expression is restricted to the proximal tubule whereas in intestine, NHE8 is found in stomach, duodenum, jejunum, ileum, and colon. NHE3 and NHE8 are developmentally regulated; NHE8 seems to be the major intestinal brush border NHE in neonates and NHE3 the predominant brush border NHE in adults [14]. In renal proximal tubules, brush border NHE8 protein expression decreases with maturation although the total amount of NHE8 in renal cortical membranes is higher in the adult compared to the neonate. Furthermore, immunostaining of adult proximal tubules revealed NHE8 in coated pit regions in addition to brush borders [53]. NHE8 KO mice do not demonstrate a metabolic acidosis and have unaltered blood pressure compared to WT mice. This is likely due to a compensatory upregulation of brush border NHE3 in the kidney [12]. In support of this, NHE3/NHE8 double KO mice had lower blood pressure and lower proximal tubular NHE activity compared to NHE3 KO mice. In the intestine, loss of NHE8 increased the susceptibility for gastric ulcers and decreased mucus secretion [181, 183]. NHE8 KO mice also exhibit disorganized mucus layers, increased adhesion of bacteria to the distal colon, and are more susceptible to mucosal injury [94]. A combination of these studies in mutant mice indicates that NHE8 plays an important role in proximal tubular Na^+ and HCO_3^- reclamation as well as in the protection of intestinal epithelia from bacterial infections. To our knowledge, no study has thus far addressed the function of intracellular, Golgi-located NHE8 in the KO mice.

SLC9A9–NHE9

NHE9 localizes to sorting and recycling endosomes and its overexpression was shown to lead to endosomal alkalinization in COS-7 cells and primary astrocytes, whereas its knockdown induced endosomal acidification in primary astrocytes [80, 115]. One exception to the intracellular localization of NHE9 has thus far been reported. In vestibular hair bundles, NHE9 is (together with NHE6) present in the plasma membrane where it serves to remove cytosolic H^+ in exchange for endolymph K^+ [65]. As in the case of NHE6, NHE9 also binds RACK1 via its cytoplasmic C-terminus and this interaction seems to affect the steady-state distribution between endosomes and the plasma membrane of these NHEs [120]. By genetic approaches, NHE9 was linked to attention-deficit hyperactivity disorder (ADHD), addiction, and mental retardation [37, 102, 109, 153]. Several rare nonsense and missense mutations not found otherwise in asymptomatic individuals were described in affected patients. An NHE9 mutation was also found in a rat model of ADHD [190]. Kondapalli et al. recently investigated the functional consequences of human NHE9 missense mutations in astrocytes [80]. Overexpression of WT but not of NHE9 mutants in astrocytes caused endosomal alkalinization and enhanced transferrin and glutamate uptake indicating that the three missense mutations found in humans are loss-of-function mutations. NHE9 is widely expressed, but the physiological role of NHE9 outside the brain is currently unknown. The phenotype of a NHE9 KO mouse has not been reported so far.

SLC9B or NHA family

Based on genomic database searches, Rao and coworkers proposed the existence of two previously unrecognized NHEs in mammalian genomes [22]. These putative NHEs had higher homology to prokaryotic NHEs than the currently known mammalian NHEs. On the basis of this homology, they were named NHA1 and NHA2, but are also known in the literature as NHEDC1 and NHEDC2 or SLC9B1 and SLC9B2, respectively. Paralogues of NHA1 and NHA2 are present in all completely sequenced metazoan genomes including: nematodes, fly, puffer fish, mouse, and human. The fruitfly *D. melanogaster* homologues of NHA1 and NHA2, CG10806 and CG31052, localize to the apical plasma membrane of Malpighian (renal) tubules. In conjunction with the V-ATPase, it has been proposed that they secrete cations into the tubular lumen. This close coupling of a primary V-ATPase with a secondarily active cation/H^+ exchange has been refered to as the "Wieczorek model" [36, 175].

The 515 amino acid long human NHA1 was cloned in 2006 [186]. Based on RT-PCR of a human cDNA panel it is exclusively expressed in testis [186]. The biologic function of NHA1 remains unknown and NHA1 has not been linked to human disease. More progress has been made in studies on the closely related NHA2 isoform. Battaglino et al. identified NHA2 by a microarray study conducted to identify genes upregulated by receptor-activator of the NF-κB ligand (RANKL)-stimulated osteoclast precursor cells [10, 135]. NHA2 was one of the most significantly upregulated genes during RANKL-induced osteoclast differentiation [11, 58, 66, 89]. While expression levels of NHA2 are by far the highest in osteoclasts, NHA2 seems to be ubiquitously expressed [48, 178]. SiRNA-mediated knock-down of NHA2 significantly inhibits osteoclast differentiation and osteoclast function in vitro [58, 89]. Recently, characterization of an NHA2-deficient (gene-trap of intron 1) mouse was reported [66]. Mutant mice were born at the expected mendelian ratio, developed normally and appeared healthy without an obvious phenotype. NHA2 protein and mRNA were completely absent

in osteoclasts derived from mutant mice. Surprisingly, NHA2-deficient mice had normal bone density. Furthermore, bone structural parameters, quantified by high-resolution microcomputed tomography, were not different from wild-type mice. In addition, in vitro RANKL stimulation of bone marrow cells isolated from WT and NHA2-deficient mice yielded no differences in osteoclast differentiation and resorptive capacity. These findings were recently confirmed independently by another group using a different, NHA2 gene-trapped mouse [29]. Even when osteoclast differentiation and activation were stimulated by ovarectomy, no difference in bone loss could be observed between the two groups of mice up to 12 weeks after the intervention (Fuster, Hofstetter et al., unpublished observations). These findings suggest that NHA2 is dispensable for osteoclast differentiation and bone resorption in mice both in vitro and in vivo, at least under the conditions employed so far.

The subcellular localization of NHA2 has been a matter of controversy [48, 66, 178]. NHA2 can be found at the plasma membrane, especially under conditions of high expression (e.g. in native osteoclasts) or exogenously in transfected mammalian cells. In addition, data from our laboratory indicate both at a functional and biochemical level that NHA2 is also present in endosomes [38, 66]. When overexpressed in NHE-deficient yeast, NHA2 conferred tolerance to Li^+ and Na^+ but not K^+, in a pH-dependent manner [48, 70, 178]. NHA2 was inhibited by phloretin but not by the classical NHE-inhibitor amiloride, even at high concentrations. Mutation of two conserved aspartic acid residues (likely involved in cation binding and/or transport) in the putative transmembrane domain 5 of NHA2 led to loss of salt tolerance in transfected yeast [178]. Rao et al. recently successfully determined NHA2-mediated phloretin-sensitive Na^+/Li^+-countertransport in MDCK cells overexpressing NHA2. While detailed kinetic studies are currently lacking, these studies clearly indicate that NHA2 is a bona fide NHE.

Based on expression pattern, genomic localization and inhibitor characteristics (amiloride resistance and phloretin sensitivity) NHA2 was proposed to be the Na^+/Li^+ countertransporter originally described in the early 1980s at a functional level, which had been linked to the development of arterial hypertension and diabetes [23, 24, 101]. In support of this hypothesis, Moe and coworkers found NHA2 to be expressed in the distal convoluted tubule of rat kidney, a renal tubular segment that is paramount for the regulation of Na^+ and blood pressure homeostasis in mammals [48]. The role of NHA2 in the kidney, however, remains unknown at the moment. A recent study looked at the role of NHA2 in the endocrine pancreas in detail [38]. NHA2 was found to be expressed both in human as well as rodent β-cells and β-cell lines. Knock-down of NHA2 in the murine β-cell line Min6 reduced glucose- and sulfonylurea-induced insulin secretion. Simultaneous overexpression of WT but not functionally dead human NHA2 rescued the insulin secretion deficit induced by knock-down of endogenous NHA2 in Min6 cells. Cellular insulin or proinsulin contents were unaltered in NHA2 deficient Min6 cells. Identical findings were observed in vitro when insulin secretion was studied with islets from two different NHA2-deficient mice (NHA2 gene-trap mice with gene-trap in intron 1 and NHA2 KO mice with targeted loss of exon 7 of the NHA2 gene) or when islets were subjected to an NHA2 inhibitor. Interestingly, islets isolated from heterozygous mice were not normal but also exhibited a secretory deficit, suggesting haploinsufficiency. Both pancreatic and islet insulin and proinsulin contents were not different between WT and NHA2-deficient mice, indicating that impaired insulin synthesis or maturation were not the cause of the insulin secretion deficit observed upon loss of NHA2. In vivo, both strains of NHA2 deficient mice displayed a pathological glucose tolerance with impaired insulin secretion but normal peripheral insulin sensitivity, compatible with the insulin secretion deficit observed in vitro. Based on subcellular fractionation and imaging, NHA2 was found to reside in endosomes and synaptic-like microvesicles in β-cells. In support of the subcellular localization, clathrin-dependent endocytosis was significantly reduced in NHA2 depleted β-cells while clathrin-independent endocytosis was not altered. Interestingly, however, loss of NHA2 had no impact on the endosomal steady-state pH in β-cells. Exocytosis and endocytosis were shown to be tightly coupled in β-cells [100, 126] and inhibition of endocytosis by various approaches reduced insulin secretion [75, 105]. Loss of NHA2 therefore may affect insulin secretion indirectly by interfering with clathrin-mediated endocytosis in β-cells, thereby disrupting endo-exocytosis coupling. Thus, although this study clearly demonstrates a role for NHA2 in clathrin-dependent endocytosis and insulin secretion in β-cells, much of the underlying mechanisms remain unknown.

SLC9C family

The sperm-specific NHE, SLC9C1, was originally identified by Garbers et al. in a spermatid (haploid cell) enriched cDNA library [171]. Mammalian sperm NHEs lack distinct orthologs in nonmammalian genomes, possess an NHE-like N-terminal domain and a long non-conserved C-terminus with similarity to the Na-transporting carboxylic acid decarboxylase transporter family (NaT-DC) [22]. SLC9C2 (also known as NHE11) is another member of the SLC9C family for which no functional data exist. Sperm NHE mRNA expression was restricted to testicular tissue by Northern blotting and dot-blot analysis of a wide array of mouse tissues [171]. When transfected in NHE-null fibroblasts, full length sperm NHE expressed poorly. A chimeric construct with the first transmembrane domain of sperm NHE replaced by that of NHE1,

however, exhibited improved expression at the plasma membrane and measurable NHE activity, suggesting that sperm NHE was indeed a functional NHE [170]. Sperm NHE KO males are infertile and display severely diminished sperm motility but normal sperm numbers and morphology [171]. Addition of ammonium chloride and cell-permeant cAMP analogues partially rescued the motility and fertility defects observed in KO sperm. Loss of sperm NHE resulted in a complete loss of full length bicarbonate-sensitive soluble adenylate cyclase (sAC) with greatly reduced bicarbonate-stimulated sAC activity [170]. sAC and sperm NHE were shown to physically interact with each other, forming a signaling complex at the sperm flagellar plasma membrane that seems to be vital for control of intracellular bicarbonate and cAMP levels, both of which are of great importance for sperm capacitation and motility [170]. Although a likely candidate, sperm NHE has not been linked to male infertility in humans.

Conclusions

As outlined in this review, NHEs are involved in a wide array of processes on a cellular as well as whole organism level. The sequencing of mammalian genomes in the last 10 years has led to the discovery of several new NHE isoforms. The physiological function of these new transporters is still largely unknown and their study will lead to important discoveries with relevance to human physiology and disease. Unfortunately, our knowledge of intracellular NHEs is still very small compared to what we know about the plasmalemmal isoforms. This is likely due to the fact that they were not discovered until recently and also due to technological limitations. Mutant mice have helped to decipher some of the physiology of intracellular NHEs but mechanistic work on a cellular level remains difficult. There is a dire need for potent isoform-specific inhibitors and improved kinetic assays to study WT and mutant intracellular NHEs. In the cases of NHE6, NHE9 and NHA2, a search for allosteric activators may lead to the discovery of compounds with pharmacological potential. Certainly, high resolution 3D structures of mammalian NHEs would be of great value not only to unravel basic transport mechanisms but also to aid in structure-based compound design.

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